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Research paper

Tracing Sox10-expressing cells elucidates the dynamic development of the mouse inner ear

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ABSTRACT

The inner ear is constituted by complicated cochlear and vestibular compartments, which are derived from the otic vesicle, an embryonic structure of ectodermal origin. Although the inner ear development has been analyzed using various techniques, the developmental events have not been fully elucidated because of the intricate structure. We previously developed a Sox10-IRES-Venus mouse designed to express green fluorescent protein under the control of the *Sox10* promoter. In the present study, we showed that the Sox10-IRES-Venus mouse enabled the non-destructive visualization and understanding of the morphogenesis during the development of the inner ear. The expression of the transcription factor Sox10 was first observed in the invaginating otic placodal epithelium, and continued to be expressed in the mature inner ear epithelium except for the hair cells and mesenchymal cells. We found that Sox10 was expressed in immature hair cells in the developing inner ear, suggesting that hair cells were generated from the Sox10-expressing prosensory cells. Furthermore, we demonstrated that scattered Sox10-expressing cells existed around the developing inner ear, some of which differentiated into pigmented melanocytes in the stria vascularis, suggesting that they were neural crest cells. Further analyzing the Sox10-IRES-Venus mice would provide important information to better understand the development of the inner ear.

1. Introduction

The cochlea and vestibule of the inner ear play important roles in hearing and balance, respectively. The structure of the inner ear can be classified into two labyrinths: the membranous labyrinth and the bony labyrinth. The membranous labyrinth is contained within the bony labyrinth, floating within a cushion of perilymph and filled with endolymph (Torres and Giráldez, 1998). In contrast, the bony labyrinth is contained within the petrous portion of the temporal bone, is filled with perilymph, and forms the lateral framework of the inner ear.

The first developmental process involving the mouse inner ear is the thickening of the ectoderm, known as the otic placode, next to the hindbrain at embryonic day 8.5 (E8.5) (Noramly and Grainger, 2002). As development continues, the placode invaginates to form the otic cup, and then the cup pinches off from the surface ectoderm to form the otic vesicle at E9.5 (Barald and Kelley, 2004). Subsequently, neuroblasts are detected in the anteroventral region of the otic vesicle, and delaminate from that region into the mesenchyme to form the neural part of the developing cochleovestibular ganglion (CVG) (Ma et al., 1998). The medial portion of the otic vesicle projects to form the endolymphatic duct at E10.75, which regulates the endolymphatic fluid volume (Salt, 2001). After the otic vesicle turns into a wide dorsal pouch region and a narrow ventral pouch region at E11.5, the delineation of the superior and posterior semicircular canals from a vertical canal plate and the expansion of a cochlear duct to half a turn occur during the next 24 h. All three canals, including the lateral semicircular canal, are well formed by E13.0. The saccule and utricle are prominent

Abbreviations: NCC, neural crest cell; CVG, cochleovestibular ganglion; IHC, inner hair cell; OHC, outer hair cell; LER, lesser epithelial ridge; GER, greater epithelial ridge.

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fixtures of the inner ear at E13.5. All primordial structures appear in their mature shapes by E15.0, and the dome-shaped canal crista ampullae are apparent and the cochlear duct expands to one and a half turns. The utricle, saccule and cochlear duct become distinct between E15.5 and E16.5. The coiling process of the cochlear duct reaches one and three-quarter turns by E17.0. Finally, the inner ear structure is almost completed at birth (Morsli et al., 1998; Kopecky et al., 2012). The complicated membranous labyrinth structures, including the cochlea and vestibule, are constructed from the epithelium derived from the otic vesicle (Torres and Giráldez, 1998; Fekete, 1999).

SoxE proteins are part of the conserved SRY-like HMG box family of transcription factors that include Sox8, 9 and 10 (Wegner and Stolt, 2005). They are known to regulate embryonic processes and determine the cell fate in various tissues (Wegner, 1999). Among the SoxE proteins, Sox10 specifically regulates both neural crest cells (NCCs) and inner ear development (Breuskin et al., 2009). NCCs, which continuously express Sox10, emerge from the dorsal neural tube and begin to migrate throughout the embryo (Mollaaghababa and Pavan, 2003; Graham et al., 2004). The NCCs differentiate into autonomic ganglia, dorsal root ganglia, melanocytes, chromaffin cells and smooth muscle cells (Le Douarin and Kalcheim, 1999). In the mammalian inner ear, the migrated NCCs differentiate into melanocytes in the intermediate cell layer of the stria vascularis, and into glial cells in the CVG (Cable et al., 1995; Britsch et al., 2001; Freyer et al., 2011). Sox10 is also expressed in various parts of the otic epithelium, ranging from the otic vesicle to the differentiated inner ear, but it is not expressed in mature hair cells (Watanabe et al., 2000).

The spontaneous mutation of the *Sox10* gene in mice leads to the development of dominant megacolon (Dom) mice, which show symptoms similar to Waardenburg-Shah syndrome type 4 in humans. Patients with this syndrome suffer from neurocristopathy appearing as sensorineural hearing loss, pigmentary abnormalities and an aganglionic megacolon (Herbarth et al., 1998; Pingault et al., 1998; Tachibana et al., 2003). Furthermore, the deficiency of the *Sox10* gene induces a shortened cochlear duct and defect of the glial cells of the CVG in the embryo, although the formation of hair cells in the organ of Corti and the neuronal differentiation in the CVG are normal (Breuskin et al., 2009; Breuskin et al., 2010). These features indicate that Sox10 is indispensable for the development of the inner ear. However, the spatial and temporal expression of Sox10 in the developing inner ears has not yet been fully investigated.

We previously developed a Sox10-IRES-Venus mouse designed to express green fluorescent protein under the control of the *Sox10* promoter (Motohashi et al., 2011). In the present study, we used these mice to investigate the Sox10 expression in the inner ear. Although the expression patterns have been reported previously (Breuskin et al., 2009; Breuskin et al., 2010), the Sox10-IRES-Venus mice allowed us to nondestructively and morphologically visualize the Sox10 expression in three dimensions throughout the development of the inner ear. In addition, this study was designed to investigate the expression patterns in more detail than the previous studies, especially during the generation and differentiation of the inner ear hair cells. As a result, we found that Sox10 was expressed in the epithelium of the membranous labyrinth, including the non-sensory otic epithelium, and that immature hair cells also expressed Sox10, together with a hair cell marker, in microscopic sections. Furthermore, we demonstrated that some of the migrating NCCs approached the developing inner ear from the dorsolateral side, and differentiated into melanocytes in the stria vascularis. The detailed and discriminative observation of the Sox10 expression provides a better understanding of the complicated development of the inner ear.

2. Materials and methods

2.1. Animals

The Sox10-IRES-Venus mice have been described previously (Motohashi et al., 2011). Briefly, these transgenic mice have a gene encoding a fluorescence protein (Venus, a GFP variant) downstream of the transcription factor Sox10, under the control of the internal ribosomal entry site (IRES). The Sox10-IRES-Venus mice and C57/BL6 mice (Japan SLC, Hamamatsu, Japan) were maintained in our animal faculty. We used C57/BL6 mice as wild-type controls in this study.

Noon on the day the vaginal plug was detected was designated as day 0.5 of gestation (embryonic day 0.5: E0.5). The developmental stages of embryos were judged by their morphological appearance as described in "The Mouse" (Rugh, 1990). All animal experiments were performed in accordance with the Regulations of Animal Experiments at Gifu University.

2.2. Preparation of the inner ear and histological analyses

Adult pregnant mice were euthanized by cervical dislocation to harvest embryos. The images of unfixed whole embryos and extirpated inner ears were taken immediately after dissection, and were examined using an Olympus SZX7 fluorescence stereomicroscope. For the histological examinations, E9.0–11.5 embryos were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 6 h at 4 °C. In the case of E12.5–15.5 embryos, the embryos were fixed in 4% PFA for 12 h. In the case of E16.5–postnatal day 21 (P21) embryos or mice, the inner ears were directly dissected and fixed in 4% PFA for 24 h at 4 °C. After fixation, the embryos or extirpated inner ears were washed in PBS and were immersed in 20% sucrose in PBS overnight at 4 °C. In the case of P21 mice, the extirpated inner ears were decalcified in 0.1 M EDTA (pH 7.4) at 4 °C for two days, before immersion in 20% sucrose in PBS. After the immersion, the embryos or extirpated inner ears were embedded in Tissue-Tec OCT Compound (Sakura Finetek), frozen at −80 °C, sectioned at 8–10 μm thickness with a cryostat (Leica CM 3050 S) and mounted onto tissue-adhering slides.

2.3. Immunohistochemical analysis

The slides were washed in PBS, made permeable by immersion in 0.1% Triton-X100 in 0.5% bovine serum albumin (BSA) for 20 min, washed in PBS and blocked in 0.5% BSA for 1 h. Primary antibodies were then added and allowed to react overnight at 4 °C. The following primary antibodies were used: mouse monoclonal anti-β-tubulin antibody (Tuj-1; 1:1000; MMS-435P, Covance, Princeton, NJ), rabbit polyclonal anti-glial fibrillary acidic protein (GFAP; 1:500; Z0334, Dakocytomation), goat polyclonal anti-Sox10 antibody (1:250; sc-17342, Santa Cruz Biotechnology), rat monoclonal anti-GFP antibody (1:1000; 04404 Nacalai Tesque), rabbit polyclonal anti-Myosin 6 antibody (1:100; 25-6791, Proteus Biosciences) and rabbit polyclonal anti-Myosin 7a antibody (1:250; sc-25834, Santa Cruz Biotechnology). After washing them in PBS again, the slides were stained with secondary antibodies for 2 h at room temperature. The following secondary antibodies were used: Texas-Red-conjugated anti-mouse IgG (1:1000; Molecular Probes), Texas-Red-conjugated anti-rabbit IgG (1:1000; Molecular Probes), Alexa-Fluor 488-conjugated anti-goat IgG (1:1000; Molecular Probes) and Alexa-Fluor 488-conjugated anti-rat IgG (1:1000; Molecular Probes). Nuclei were stained with DAPI (Invitrogen). The sections were rinsed in PBS, mounted in Vectashield and then examined with a confocal microscope (Olympus Fluoview FV1000).